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An Enzyme Method Of Clearing and Staining Small Vertebrates

By William Ralph Taylor
Associate Curator, Division of Fishes

Introduction

Cleared (transparent) and stained specimens are used widely for osteological studies of vertebrates. Many such specimens have been prepared by the Schultze method (Schultze, 1897) or modifications of that method (Davis and Gore, 1947; Evans, 1948; Hollister, 1934; Jensh and Brent, 1966). The Schultze method and its modifications chiefly involve tissue maceration in solutions of potassium hydroxide or sodium hydroxide, staining the bones to give them maximum visibility, and immersion of the specimen in a liquid with an index of refraction similar to that of the cleared tissues.

The method is successful when it is used on fresh or newly preserved specimens, but numerous workers have encountered considerable difficulty in producing good transparent specimens by the above methods after specimens have been stored in various preservatives for several years. Such specimens may remain opaque because of dark stains in the flesh or they may swell and the membranes rupture during the clearing process.

The new method described in this paper substitutes enzyme digestion for the alkaline maceration. It offers several advantages, including ease of clearing the old material. Other methods such as cleaning by dermestid beetles or flesh maceration by boiling are usually not desirable in the preparation of skeletons from small, delicate specimens because the bones may be lost, damaged, or distorted.

Purified trypsin powder was first tested, in distilled water with a trace of potassium hydroxide, on formalin-fixed specimens that had been stored for several years in ethyl alcohol. These tests were slow in producing transparent specimens. The odors given off from the solutions were very disagreeable but the results indicated that enzymes should be explored.

Because the initial preparations remained in good condition during two years in glycerin, tests of various enzyme-buffer solutions were undertaken. Of these, the sodium borate (borax) solution was found to be superior. It supports the most rapid enzyme activity while maintaining a relatively stable and desirable pH over a long period of time, and it inhibits bacterial growth that results in disagreeable odors.

Due to the great variation in specimens and in their preservation, results are not always predictable. The enzyme method generally produces good transparent study specimens from individuals that have been properly preserved and cared for prior to enzyme treatment, except where thick or dense connective tissue is present as in some small species and most large specimens. The enzymes are less harmful to specimens than strong alkalis because the digestion removes muscle tissue and stains and permits slight collapse of membranes over the evacuated areas rather than extensive swelling with its splitting and distortion or disintegration of tissue. Specimens will fall apart occasionally or disintegrate during enzyme digestion as well as in caustic potash solutions. This appears to result from faulty preservation that permitted prior connective tissue alteration or digestion. These specimens are more likely to be usable following digestion than following caustic treatment because the former frees the specimen of bulky and weighty materials that cause damage when it is moved.

I have used the enzyme method successfully to clear several hundred specimens of fishes of more than 100 species, belonging to 40 families, and also several specimens of amphibians, reptiles, and mammals. The method has been of value in restoring glycerin specimens of fishes that had been treated previously with potassium hydroxide. The enzyme method would thus appear to be useful as a means of clearing small specimens of all vertebrates.

I have not tested proteolytic enzymes other than those of pancreatic origin. Their maximum activity is usually at a pH below 7.5 or in

strong acids, which may be damaging to skeletal tissue. The pancreatic extracts are inexpensive, breaking down denatured proteins, especially those of muscles, and work best in a favorable, slightly alkaline medium.

There are few published accounts on the use of enzymes in the preparation of osteological material. Moser (1906: summarized in Piechocki, 1961, p. 285) recommended a solution of potassium carbonate and trypsin powder for clearing embryos. The method has remained relatively unknown. In my tests, clearing was slow, solutions rapidly became acid, and there was loss of bone minerals. Piechocki (1961, p. 228) summarized reported uses of papain, pepsin, and trypsin for cleaning flesh from skeletons. The papain and pepsin methods involve the use of acids and were not tested because of probable damage to bones. The trypsin methods include the use of sodium sulphide and higher temperatures than I have employed. My results indicate that sodium sulphide-enzyme solutions have less advantage than borax-enzyme solutions for both clearing and defleshing preserved specimens. Some damage to specimens occurred from rapid pH drop to acid conditions. True (1948) described a slightly acidified pepsin solution for removal of fats and proteins before staining.

I made frequent checks on the pH of all solutions during the process of working out the method. The readings were obtained from a Beckman pH meter, Model G, calibrated at pH 7.0 by use of the "Fisher Standard Buffer Solution" specified as pH 7.00 ± 0.02 at 25°C (monopotassium phosphate-sodium hydroxide buffer, 0.05 Molar). In comparative tests, specimens of one species with presumed identical preservation were used, and specimens of equal sizes were selected insofar as possible. They were placed in solutions of equal volume with similar enzyme content. All reactions were at room temperature, which varied from 22°C to 27°C.

I am especially appreciative for the laboratory assistance of Kenneth Karb, Phyllis Herrington, and Fanny Phillips, and the encouragement received from the staff of the Fish Division in the development of the process.

Materials Required

1. Alizarin red S powder. This material should be one of those certified by the Biological Stain Commission as satisfactory for staining bone. A few grams will be sufficient for several years use in most work.

2. Potassium hydroxide (KOH), U.S.P. grade. The pellet form is the most satisfactory in rapid preparation of solutions.

3. Distilled water.

4. Hydrogen peroxide (H_2O_2), U.S.P. grade. A 3 percent solution keeps better than stronger solutions and has been found satisfactory.

5. Borax or sodium borate powder (sodium tetraborate, $Na_2B_4O_7 \cdot 10H_2O$), U.S.P. grade or better is preferred.

6. White glycerin, U.S.P. grade or better is preferred. The liquid should be obtained and stored in glass containers as it becomes colored in contact with metal.

7. Trypsin powder (purified).[1] This material may be sold also as pancreatic protease and pancreatin. It typically contains a wide variety of enzymes as well as trypsin. The commercial preparation must be free of collagenase contamination and must have been purified to the extent that it is free or nearly free of elastase. One method of effective removal of elastase from pig pancreas preparations that resulted in a good yield of trypsin and chymotrypsin was described by Kunitz and Northrop (1936); see also Baló and Banga (1950). Highly purified and concentrated trypsin preparations are expensive and unnecessary. Extensive purification may reduce or eliminate desirable enzymes that are produced by the pancreas, particularly the chymotrypsins and the wide variety of other proteases as well as lipolytic and diastatic enzymes. The enzyme potency is often expressed as a ratio. The figure 1:80 usually means that one part will digest 80 parts casein when measured by the specified pancreatin test. Other specifications may include the activity of individual enzymes expressed as units per gram.

The dry powder is stable and may be stored at room temperature for a considerable length of time. To insure stability and to reduce possible bacterial multiplication, however, I suggest keeping it in a tightly closed container under refrigeration. One hundred grams is sufficient to clear nearly 100 small specimens, if they are properly prepared.

8. Thymol, N.F. crystals ($C_{10}H_{14}O$).

9. Concentrated formaldehyde solution, about 40 percent by volume, U.S.P.

10. Ethyl alcohol (ethanol), 95 percent, U.S.P. (optional; used in step 10 of "Method" section). Absolute (100 percent) ethyl alcohol is desirable.

11. Xylene (xylol) (optional; used in step 10 of "Method" section).

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[1] I have made extensive use of Fisher Scientific Company's "Purified Trypsin Powder" (1:80 N.F.), Catalog Number T-360. Similarly good results have been obtained from Difco Laboratories' "Trypsin, Difco Certified" (1:250 N.F.), Code Number 0152.

Preparing Stock Solutions

1. Saturate distilled water with sodium borate powder. Mix thoroughly and allow to settle until supernatant is clear. A large volume of the solution can be mixed well in advance and used as needed. When preparing a new supply, break the caked borax at the bottom of the container and add sufficient new powder to insure saturation.

2. Prepare a solution of one-half to one percent potassium hydroxide in distilled water.

3. Prepare the enzyme buffer solution by mixing three parts of the clear upper liquid from the saturated sodium borate solution (1) with seven parts distilled water if the temperature of the saturated borate solution is in the range of 22° to 27°C (about room temperature). This concentration (30 percent saturated borax solution) is estimated to be approximately 0.9 to 1.0 gram sodium borate per 100 ml. of the solution at 25°C.

If bacterial contamination takes place in the clearing process at this strength and the original borate solution (1) is fully saturated, the amount of saturated borax may be increased slightly in new solutions. The solubility of borax in water varies materially with the temperature. The following quantities of saturated borate should be used in preparing enzyme buffer solutions when the saturated solution is at the temperature indicated, based on the saturation rate ("Handbook of Chemistry and Physics").—10°C: use 6½ to 7 parts saturated borate solution and 3 to 3½ parts distilled water for 10 of solution; 15°C: 5 to 5½ parts borate; 20°C: 3½ to 4 parts borate; 30°C: 2½ parts; 35°C: 2 parts; 40°C: 1 part borate in 6 of solution; 45°C: 1 part borate in 8 of solution.

4. Mix the following solutions of glycerin and potassium hydroxide solution (2) or distilled water:

a. 40 percent glycerin and 60 percent potassium hydroxide.

b. 70 percent glycerin and 30 percent potassium hydroxide.

The Method

In the following step-by-step process it is necessary to avoid contamination of specimens and materials in order to curtail undesirable organic growth in the solutions. Do not expose specimens to high temperatures. A maximum of 30°C is recommended for most fishes. Slightly higher temperatures should not damage warmblooded vertebrates and those that have lived in a hot environment.

1. Kill specimens in 10 percent formalin (about 4 percent formaldehyde) and leave in this solution for one week to 10 days. This fixing solution consists of one part by volume of concentrated formal-

dehyde solution plus nine parts water and specimens. Buffer in about one day by adding one-half teaspoon of borax powder per quart of solution. Open the abdominal cavity and the gut to expose them to the preservative. Such structures as fur and feathers, which may be difficult to remove later, should be removed at this time. The viscera, most scales, and many other undesired parts preferably are removed after staining to prevent loss of small bony structures.

The best preservation results from killing the animal in the fixing solution. Dead but unpreserved animals should be fixed without delay. Whether they will yield satisfactory specimens depends upon the length of time they have been dead and upon their prior treatment. Short delays, even of less than an hour after death, and exposure to high temperature before fixation may result in specimen contamination, decomposition, and noticable connective tissue breakdown. The destruction of tissues continues slowly when the specimen is under refrigeration and, to some extent, when the specimen is frozen, although some specimens that were frozen for several days have turned out satisfactorily without preservation.

Small specimens that have died immediately prior to the enzyme treatment sometimes result in good preparations without preservation, but fixation is more reliable. If they are not fixed, they should be eviscerated and subjected to the clearing process, beginning with step 3 without delay.

Specimens stored in isopropyl or ethyl alcohol require no further preliminary preparation. Those that were initially fixed in alcohol or that exhibited spoilage before formaldehyde fixation may disintegrate in digestion. Specimens damaged, twisted, or distorted when fixed become good cleared examples that often are not distinguishable from those that were preserved in a more normal position; they usually are chosen for clearing in order to retain the better specimens for other purposes.

2. Pour off formalin or any preserving solution containing the odor of formaldehyde and soak the specimen in repeated changes of water until the formaldehyde is removed. Formaldehyde as well as some metals and some apparent formaldehyde derivatives that are not soluble in water or alcohol inhibit the enzyme reaction (see further comments in step 6). If the specimen is not to be cleared immediately, it should be stored in a 70 percent solution of ethyl alcohol.

3. Bleach the specimen in a solution of 10 to 20 percent stock hydrogen peroxide and 80 to 90 percent stock potassium hydroxide. Because the reaction is enhanced by light, it may be placed in a transparent container over a light box or beneath a lamp. Body cavities containing heavily pigmented membranes should be opened

and their organs loosened or spread to allow access of the bleaching solution.

Bleaching at this time is important. If done later, the evacuated body cavities tend to become filled with bubbles. Remove bubbles, if possible, following bleaching. A small vacuum pump is useful for this purpose. If the bubbles cannot be removed, they will slow the clearing, but they will disappear in a closed actively digesting enzyme solution in a few days.

The enzymes do not extensively remove color pigment (chromatophores and guanin) from specimens, but dark pigment is noted to flow out and materially darken digestion solutions subsequent to partial bleaching. Complete dissolving of the pigment from a specimen, thus, may not be necessary. Bleaching is continued until all dark areas have become decidedly pale, the time varying from about half an hour for poorly pigmented specimens to several hours for specimens with thick layers of pigment (e.g., atherinid fishes).

4. Place the specimen in a buffer solution, adequate to cover it, of volume 10 to 40 times that of the specimen. If the specimen has been in glycerin, use the methods described in the section "Glycerin Specimens" (p. 10).

Large reservoirs of liquid, other than enzyme buffer solution, which are in the evacuated body cavities of a partially digested specimen, should be diluted by placing the specimen in a borax solution for several hours prior to placing it in the enzyme buffer solution. Excess water will dilute the buffer solution; hydroxides, isopropyl alcohol, and ethyl alcohol will tend to elevate the pH of the solution above the optimum. Traces of these liquids that may cling to the outside of a specimen can be ignored.

Excessive volume of the buffer solution will dilute and slow enzyme activity. Insufficient volume will permit a rapid pH drop and resist diffusion of digestion products from the specimen. The adequate volume of buffer solution to use appears to be 10 to 40 times the volume of the specimen. A minimum of 200 ml. of the solution has been found satisfactory for very small specimens.

Several specimens, if properly fixed, may be placed together in one solution for clearing.

Glass and plastic containers both have been used for the process with good results. Containers that can be sealed are preferred as they tend to limit bacterial contamination, permitting longer use of a solution.

5. Add the enzyme (trypsin powder). One-fourth of a teaspoon (about 0.45 grams) of enzyme is adequate for most specimens weighing up to 1 or 2 ounces and requiring 400 ml. of liquid or less for immersion. Larger specimens may require more enzyme. Tiny specimens may

be cleared with materially less enzyme, but it is desirable to reduce the volume of buffer solution if a very small amount of enzyme is used. Mix well but do not froth, close the container, and set the solution aside at 20°C to 30°C (most room temperatures are satisfactory) to clear the specimen. Higher temperatures for digestive activity are not recommended as they invite increased bacterial activity and may denature connective tissue in lower vertebrates.

Enzymes lose potency in the solution and should be added only at the time they are to be used.

6. Leave the specimen in digestion until one-tenth to one-fourth of the muscle tissue remains. Change the digestion solution every week to 10 days. If specimen has been in glycerin, the first digestion solution must be changed in four or five days; the second and subsequent solutions may be retained for a week to 10 days.

The digestion solution may be cloudy (due to some enzyme preparations) or clear, and it tends to remain so unless coagulated or precipitated by some extraneous materials. It will darken gradually with release of stains and pigment from the specimen. Clearing of the specimen will first be noted along the edges of the body and in the thinner portions such as the abdominal wall, the caudal peduncle, and the limbs. The specimen will lose rigidity, becoming very flexible as digestion proceeds. Some small specimens can be cleared adequately in one or two days; larger specimens and those containing enzyme inhibitors may require weeks or months in the solution to become sufficiently cleared.

Although a solution may remain active for several weeks, the maximum activity appears to decline after a week to 10 days. As a general rule, a solution should be discarded before it has been used two weeks to avoid bacterial growth and excessive pH drop.

The odor of the solution will initially be similar to that of trypsin powder. Gradually, inoffensive odors due to the digestion may become evident. Specimens that have been stored in such materials as oil of cloves, isopropyl alcohol, and thymol, even though washed to remove these materials, may impart their strong odors to the solution. Growth of bacteria may result in very offensive odors of putrefaction from the solution, in which case the solution should be discarded. The specimen is then cleaned by placing it in 70 percent ethyl alcohol or stock potassium hydroxide solution for a short time and, beginning with step 4, is returned to a new digestion solution in a clean container. If the odor of putrefaction is again produced, the process must be repeated but an increase in the amount of saturated borax solution is suggested (stock solution 3).

When the specimen is nearly transparent—having lost three-fourths or more of its dark muscle tissue and retaining only a few

small areas of yellow or brown muscle tissue as remnants of larger or thicker muscle masses—it should be stained (step 7).

Specimens may not clear or may clear slowly because of enzyme inhibitors. Common indicators of inhibition are a musty odor, due to formaldehyde, or extensive bottom precipitate with the tendency of a cloudy solution to become clear. A bath in stock potassium hydroxide solution for one or two days is suggested to aid in removing the inhibitor. This is followed by repeating the digestion process, beginning with step 4. The purpose of the potassium hydroxide treatment is to remove water and alcohol-insoluble enzyme inhibitors. Alternation of potassium hydroxide and digestion treatments may be repeated until the specimen becomes adequately clear, processes that may require several weeks or months to complete.

Some specimens, typically large individuals but also some small ones, have thick connective tissue and remain opaque even in liquids with a high refractive index. They can be made usable by cutting away the undesired connective tissue after completion of the clearing and staining process.

Poorly preserved specimens may quickly disintegrate. To stop or slow the digestion, about 10 percent formalin may be added to the solution, or the specimen may be transferred (carefully) to 70 percent ethyl alcohol.

Providing the solution has had little digestive activity and has a relatively high pH, no damage to adequately fixed specimens results from their retention in a digestion solution for several days or weeks following clearing. Unless extensive digestion takes place, the 30 percent saturated borax solution is adequate to maintain a satisfactory pH of 7.5 or higher for several weeks in the presence of the enzyme.

7. Prepare a stain solution by mixing a very small quantity of alizarin red S powder in sufficient stock potassium hydroxide solution for specimen immersion. Add alizarin until a deep purple or the desired color intensity is reached. Place specimen in stain solution and let it remain until the bones have become adequately stained.

No advantage is evident in using the specially prepared stain solution described by Davis and Gore (1947, p. 8) or Hollister (1934, p. 92). A solution once prepared may be retained and used to stain additional specimens.

An alcohol stain, prepared by dissolving alizarin red S in 70 percent alcohol and made slightly alkaline by the addition of a few drops of stock potassium hydroxide solution will stain the specimens that started to disintegrate in digestion without further extensive harm. After staining, proceed with them to step 12.

8. Remove specimen from stain solution, rinse in distilled water, and then eviscerate, remove scales and all undesired parts.

9. Rinse specimen in distilled water or soak in borax solution, if large quantities of alkali are suspected to be present in the body cavities, and then return to the digestion solution if it is usable; otherwise, repeat the process, beginning with step 4. Continue digestion until all areas of muscle tissue have disappeared.

Completion of the clearing process following staining is suggested because virtually all of the stain embedded in the soft tissues is removed along with the remaining areas of dark brown or yellowish muscle tissue.

10 (optional). Some oils and fats that remain after the digestion process may be removed by gradually transferring the specimen through a series of ethyl alcohols in varying strengths, beginning with about 40 percent, thence through 70 percent, 95 percent, to absolute alcohol (if available), and then to xylene. After the xylene, the specimen is transferred downward in the alcohol series to water and washed until all traces of the xylene are removed.

11 (optional). Guanin or guanin-like substances may be removed by soaking the specimen in a solution of 2 to 4 percent potassium hydroxide for several days. Guanin is seldom dense, opaque, or troublesome in preserved specimens, especially those preserved in formalin. It is almost insoluble in water, only slightly soluble in alcohol, but it is freely soluble in dilute acids and aqueous potassium hydroxide solutions.

12. Following digestion, many specimens are adequately transparent for study in alcohol. To attain uniformity and to avoid storage problems, specimens are worked into glycerin through a series beginning with 40 percent glycerin in distilled water or in stock potassium hydroxide solution (stock solution 4a), followed by 70 percent glycerin (stock solution 4b) and full strength glycerin. The potassium hydroxide and glycerin mixtures are preferred to create a slight residual alkalinity for long-term storage of specimens. A few crystals of thymol are added to the final glycerin to prevent growth of microorganisms, as the digestion products are an ideal medium for such growth.

For final storage, the specimen is placed in a glass jar with a bakelite top. The jar size should be sufficient to allow complete coverage of the specimen by glycerin but sufficient space between the glycerin and top so that it can be screwed on loosely without danger of glycerin spillage. Metallic containers and tops should be avoided as glycerin reacts in contact with metal, often resulting in colored glycerin.

GLYCERIN SPECIMENS.—Specimens that have not been successfully cleared by any of the modifications of the Schultze method (Davis and Gore, 1947; Evans, 1948; Hollister, 1934) may be improved or

completely cleared by enzyme digestion. These specimens are often brown and opaque, especially after long storage in glycerin. Frequently the skin has been split, the tissues are swollen, and parts may remain attached only by narrow strands of tissue. With careful handling and clearing, these poor specimens can often be made very useful unless the fragmentation of connective tissue by the alkali treatment has been extensive. In digestion, much of the bulky material is removed and the swelling is replaced by the collapse of elements, which approximates their normal position. I have cleared many specimens successfully with enzymes, after they had been macerated for varying lengths of time in potassium hydroxide solutions ranging from 0.5 percent to 9 percent, without damage beyond that due to the alkali.

The suggested procedure:

1. For average specimens, dilute the original glycerin and thymol mixture to one-half by addition of distilled water. Let stand for about half a day. Tough specimens may be removed from the original glycerin directly to distilled water or borax; on the other hand, very delicate and badly macerated specimens should have their glycerin diluted more gradually.

2. Place specimen in one or two (depending on size) changes of distilled water or borax solution for one-half day or longer to remove glycerin.

3. Clear specimen by digestion, following the appropriate steps 4 through 12 as described in the preceding section, and repeat as necessary. *Do not* retain the initial digesting solution longer than four or five days as, owing to the presence of glycerin, the first solution rapidly putrefies and becomes acid. Since nearly all of the glycerin and much of the muscle tissue will have been removed in the first digestion solution, the second digesting solution will be more stable and may be retained longer.

Reactions of the Pancreatic Enzymes

The powdered enzyme preparation is usually slightly acid. The proteolytic enzymes present are active from slight acidity to moderate alkalinity, with pH 7.5 to pH 9.0 usually cited as the optimum for trypsin and chymotrypsin.

These enzymes break down proteins, especially denatured proteins, converting them to polypeptides and amino acids; thus, denatured connective tissue is digested. Elastin is not easily denatured but is digested by elastase. For this reason, it is advisable to determine that elastase has been eliminated from the pancreatic enzyme preparation. The other important connective tissue component, collagen,

changes to gelatin with heating and is then subject to general proteolytic degradation. Such materials as acids, some chlorine compounds, and strong bases may extensively damage organized collagen fibers and connective tissue structures; degradation in trypsin results from treatment with some of them. Collagenase, which digests native collagen, is apparently not produced by the pancreas. An extensive account of enzyme breakdown of connective tissue is given by Mandl (1961). Limited carbohydrate and fat digestion may be expected.

In solution the enzymes become inactive, and the rate of inactivation of trypsin increases, with increase in pH and temperature, to a rather rapid loss of activity when the trypsin is in moderate to strong alkaline solutions (Tauber, 1950, p. 18).

The reaction rate of enzymes depends partly upon the nature of the substrate, the temperature, and the inhibitors present in solution. Inhibition is increased with increase in acidity or alkalinity or with concentration of a nonsubstrate substance beyond the optimum. Tauber (1950, p. 18) has listed a number of inhibitors of trypsin that are important in this method: charcoal, unsaturated fatty acids, tryptic digests of proteins, pancreatic trypsin inhibitor, hydrogen sulphide, alcohol, formaldehyde, thymol, alkaloids, glycerol, fats, sugar, a number of inorganic salts including heavy metals, ionizing radiation (x-rays, ultraviolet light), and bacteria.

The optimum temperature for digestion is probably near the body temperature of the animal from which the enzymes were obtained. At both lower and higher temperatures the activity is slowed, and at higher temperatures the enzymes may be denatured. To prevent thermal denaturization of connective tissue, avoid heating specimens. Temperatures of 30°C or below are recommended for fishes; somewhat higher temperatures will probably not harm reptiles, birds, or mammals (see Takahashi and Tanaka, 1953; Takahashi and Takei, 1954; Takahashi and Yokoyama, 1954).

The pH of the active digestion solution dropped in all cases that I observed, the rate depending upon the enzyme activity and the quantity of buffer solution used. After most digestion solutions have become virtually inactive, they cannot be restored to any worthwhile degree of effective activity by the addition of either an alkaline solution (to elevate pH) or by the addition of more enzyme. But the relatively inactive enzymes in alcohol solutions may be activated to some extent, even after several months, by slight dilution of the alcohol and by adjustment of the pH toward the optimum.

A number of buffers suitable for enzymatic and histochemical studies are described by Gomori (1955). Tests of a number of alkaline materials, including common chemicals, some of the buffers

described by Gomori, and many chemicals used in specimen preservation, were made by me. None showed the digestive activity and pH stability of a 30 percent saturated borax solution, many developed strongly offensive odors, and some appeared to be moderate to strong enzyme inhibitors. Very little digestive activity was observed in solutions with pH readings above 10.0, but with a gradual drop in pH the activity often increased.

Although sodium chloride is an elastase inhibitor (Mandl, 1961, p. 233), it slowed the reaction rate, and no effect on connective tissue from its use was observed. Distinct loss of alizarin staining minerals from fin rays was observed after digestion in the following (all with rather rapid drop to low pH readings, the initial pH followed by final pH in parentheses): dilute trisodium phosphate (10.3 to 4.8); dilute hexamine (hexamethylenamine) (7.4 to 5.5); dilute sodium hydroxide (various dropping to 5.0–6.0); distilled water (6.4 to 5.2–6.0); 0.3 percent potassium carbonate, recommended by Moser (1906) for clearing embryos by trypsin digestion (10.2–10.5 to 5.6–6.5); dilute sodium sulphide recommended by Piechocki (1961, p. 228) for defleshing specimens (10.0 to 6.5). Similar specimens in solutions with pH readings remaining above 7.0 did not undergo this loss. No significant damage to connective tissue by digestion was observed in specimens previously soaked in potassium or sodium hydroxide, but many specimens that had been preserved and allowed to remain in unbuffered formalin exhibited loss of the alizarin staining minerals.

Specimens removed from formalin preservative without washing digested very little, if any; and, in borax solutions, the enzyme coagulated and precipitated at the bottom. These specimens could be cleared adequately only after repeated changes of digestion solution or after being washed in water for several days to remove the formaldehyde. Specimens that had remained in formalin storage for a lengthy period of time generally took longer to clear than did specimens stored in alcohol although the formaldehyde had apparently been removed by soaking in water. Likewise, specimens in alcohol solutions that contained a slight odor of formaldehyde required prolonged clearing.

The presence of 0.033 percent formalin in the borax buffer solution prolonged the clearing time for a specimen several times that of solutions without formaldehyde, requiring several changes of digestion solution, but 0.1 percent or more formalin in the buffer (in which formaldehyde odor could not be detected) appeared to prevent all digestion.

Sodium borate solutions were prepared by using measured quantities of borate and distilled water or by saturating distilled water with borate powder and diluting with distilled water at room temperatures

of 22° to 27°C. The percentages used in this paper are percentages of saturation at these temperatures, i.e., the volume of saturated borate solution per hundred of the final enzyme buffer solution. The saturated borate solutions ranged from pH 9.1 to 9.3. In 200 ml. of enzyme buffer solution the addition of about one-half gram of enzyme powder resulted in slight but obvious drops in pH, in solutions below 25 percent saturated, or 0.8 gram borax per hundred. Solutions in the range from 5 to 20 percent saturated often became disagreeably contaminated with bacteria in 10 days. Sometimes, but very infrequently, solutions from 25 to 33 percent saturated developed disagreeable odors in this period, perhaps owing to either excessive initial bacterial contamination from unclean containers or to a failure to obtain complete saturation of the initial borate solution. Generally, solutions from 30 percent saturated upward are expected to prevent excessive bacterial growth over a period of time sufficient to obtain the maximum digestive use of an enzyme.

The rate of pH drop depends on the relative volume of the solution, the quantity of buffer in solution, and the digestive activity. Active 5 percent saturated borate solutions frequently dropped from pH 8.8 or 8.9 to pH 6.7 or lower in 10 days without adequately clearing specimens. Similar 20 percent solutions dropped from pH 9.1 or 9.2 to pH 8.2–8.5, and 50 percent solutions dropped from pH 9.2 to pH 8.9–9.0 in the same time, but both with virtual clearing of specimens. Solutions containing 15 percent or less saturated borate or 60 percent or more took longer consistently to clear specimens than did solutions between these ranges. Saturated solutions of borate required the longest activity. Little difference in clearing rate was noted in 20 to 50 percent saturated solutions, with the best results believed to have been in those 25 to 33 percent saturated. Numerous tests of the 30 percent solution indicate that it remains desirably stable and infrequently drops below pH 8.0, even after several weeks.

Solutions of 2 to 6 grams borate per thousand, corresponding approximately to solutions 20 percent saturated or less, gave similar rapid drops in pH and quickly developed extensive bacterial cultures without adequate specimen clearing. Those with 8 to 16 grams borate per thousand gave results similar to the 25 to 50 percent saturated solutions. Borate solutions of 18 to 22 grams per thousand, corresponding to about 55 to 70 percent saturated, permitted slow clearing, similar to solutions with 6 grams or less. Borax-enzyme solutions of volumes 10 to 40 times that of the specimens have generally been found adequate. Excess volume of solution, several hundred times that of the specimen, resulted in a dilution of enzyme, and it nearly doubled the specimen clearing time. On the other hand, an inadequate volume of solution quickly becomes saturated with digestion products,

with accompanying drop in pH and curtailment or termination of digestive activity.

The 30 percent solution has proven superior to all other materials tested in speed of enzyme activity, in maintaining a relatively stable pH, and in preventing or curtailing bacterial activity.

Specimens containing glycerin, when cleared in this enzyme buffer solution, produce a rapid pH drop that may descend to the undesirably acid pH 6.0 in less than one week if the glycerin is not carefully washed from the specimen.

Summary of the Clearing and Staining Method

1. If specimen is not already in alcohol, preserve it in 10 percent formalin for one week to 10 days.
2. Soak all formaldehyde from specimen.
3. Prepare a saturated solution of sodium borate in distilled water.
4. Prepare a stock solution of one-half to one percent potassium hydroxide in distilled water.
5. Bleach specimen in about 10 percent stock hydrogen peroxide and 90 percent stock potassium hydroxide solution.
6. Prepare an enzyme-buffer solution of 30 parts saturated sodium borate solution (supernatant) and 70 parts distilled water of volume equal to 10 to 40 times that of specimen.
7. Place specimen directly in buffer solution or, if necessary, remove any large quantities of alcohol, water, or potassium hydroxide from specimen by soaking it in a borax solution, and then place it in buffer solution.
8. Add about one-fourth teaspoon of enzyme to solution for small specimens, slightly more for large specimens; mix and place aside to clear at about 25° C.
9. Change solution (steps 6 to 8) in one week to 10 days, and repeat until specimen has only a few small areas of opaque muscle tissue remaining.
10. Stain specimen in a solution of stock potassium hydroxide and alizarin dye.
11. Remove viscera and undesired parts.
12. Return specimen to digestion solution (steps 6 to 9) until completely cleared.
13 (optional). Remove oils by working specimen through alcohol series into xylene. When oils have been removed, work back through alcohol series and wash to remove all xylene.
14 (optional). Dissolve dense deposits of guanin in a 2 to 4 percent aqueous solution of potassium hydroxide.
15. Work specimen gradually into full glycerin and add a few crystals of thymol.

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